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14. ABSTRACT

Mitogen activated protein kinase kinase 4 (MKK4), a member of the stress activated protein kinase signaling pathway, acts as a metastasis suppressor protein in ovarian cancer. Introduction of MKK4 into a highly metastatic human ovarian cancer cell line reduces the number of overt metastases by 90% and prolongs animal survival by 70%. Our specific aims in this proposal included determining the mechanism by which MKK4 mediated metastasis suppression and determining whether introduction of MKK4, a potent activator of the JNK pathway, in conjunction with chemotherapeutic agents known to activate JNK would potentiate MKK4's effects on survival and JNK activation. Our key findings during the period of the proposal included the following: 1) MKK4 mediates metastasis suppression via the p38 pathway 2) MKK4 does not mediate metastasis suppression via the JNK pathway 3) MKK4 kinase activity is required for MKK4 induced metastasis suppression. In the past year, our findings have included 4) MKK4 expression does not augment the effects of either cisplatin or paclitaxel on metastases in vivo. 5) There do not appear to be any differences in JNK or p38 activation levels in metastases taken from mice injected with MKK4 and treated with chemotherapy (cisplatin or paclitaxel) over chemotherapy alone.

15. SUBJECT TERMS
Metastatic colonization, ovary, MKK4, metastasis

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I. Introduction

Ovarian cancer is the most lethal of the gynecologic malignancies. In 2006, according to American Cancer Society statistics, it is expected to affect 20,180 women with an attendant 15,310 deaths, making it the fifth most common cancer to contribute to the female death rate.[1] The vast majority of women, over 70%, will be diagnosed with widespread metastatic disease. Attempts at early detection of ovarian cancer have met with little success. With aggressive surgical cytoreduction and the identification of more effective chemotherapeutic agents, the 5 year survival rates have improved over the past 20 years from approximately 35% to 50%;[2] however, because patients eventually develop chemotherapy resistant disease, the long term overall survival remains low at 20%. To improve patient survival, we must improve our understanding of the mechanisms governing metastasis.

Mitogen-activated protein kinase kinase 4/c-Jun NH2-terminal kinase-activating kinase/stress-activated protein/ERK kinase 1 (hereafter referred to as *MKK4*) has been identified as a metastasis suppressor gene in prostate cancer.[3,4] Metastasis suppressor genes are defined as genes that encode proteins that regulate metastatic tumor growth without necessarily affecting primary tumor growth. We have previously shown that MKK4 protein is downregulated in 68% of clinical ovarian cancer metastases. In addition, we have shown that introduction of MKK4 into a highly metastatic ovarian cancer cell line (SKOV3ip.1) decreases the number of visible metastases by an average of 90% when compared to their control counterparts. Finally, animals injected with SKOV3ip.1-HA-MKK4 survive longer: a mean number of 63 days as compared to 37 days in animals injected with vector control cells, a prolongation in survival of nearly 70%.[5] This effect on metastasis suppression is eventually overcome with time suggesting that MKK4 mediates its effects during metastatic colonization, the point at which disseminated cancer cells lodge and grow at secondary sites of disease.

Because MKK4 is a member of a well-characterized signaling pathway (the stress activated protein kinase signaling pathway, SAPK) **(Figure 1),** we set out to explore two specific aims in this proposal: 1) to determine whether introduction of MKK4 reconstitutes a signaling cascade that inhibits metastatic colonization and 2) to determine whether the combined effects of MKK4 and chemotherapy, both known to activate the JNK pathway, potentiate the effects of MKK4 alone. An improved knowledge of signaling pathways requisite for cancer cell growth and metastasis will allow for the development of targeted therapies and, perhaps, the identification of molecular markers that will predict response to chemotherapy in ovarian cancer.

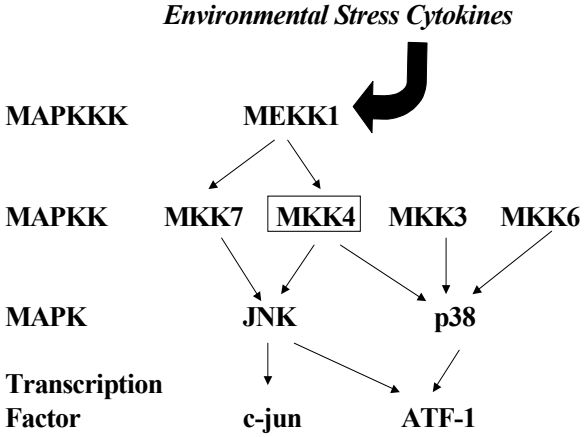


Figure 1. Major components of the SAPK signaling cascade. Signals from the cell surface are transmitted by a series of protein kinases. Activation of MEKK1 results in phosphorylation of MAPKKs (MKKs 3, 4, 6 and 7) which phosphorylate either JNK (MKK7), p38 (MKK3 and MKK6) or both (MKK4). Phosphorylated p38 and JNK activate/repress trasnscription factors.

II. Body

In our proposal, we set out to explore two specific aims. In specific aim 1, our hypothesis was that ectopic expression of MKK4 would reconstitute a context dependent signaling cascade that would inhibit metastatic colonization. To this end, we proposed to test 1) whether an upstream activator of MKK4, MEKK1, would suppress metastatic colonization 2) whether MKK4 kinase activity was required for metastasis suppression and 3) whether MKK4 was exerting its effect on metastasis suppression via the JNK or p38 pathways as outlined in **Figure 1**. Our model utilizes a highly metastatic human ovarian cancer cell line, SKOV3ip.1. After ectopic expression of components of the SAPK pathway in SKOV3ip.1, mice are injected intraperitoneally with 1 x 10^6 cells or pLNCX2 vector and the number of metastases, greater than 1 mm, counted either 20 or 30 days post-intraperitoneal injection (dpi).

1. MEKK1 as an upstream activator of MKK4. The functional evaluation of MEKK1, a candidate MAP3K involved in MKK4 mediated metastasis suppression, was complicated by the lack of specificity of the MEKK1 antibodies available to us as well as difficulties in manipulating MEKK1 wild type and mutant constructs. These persistent difficulties prompted us to re-evaluate our approach to this aim. To this end, we turned our attention to the identification and testing of other MAP3K's that might activate MKK4 including Tao, Tak1, MLK3, and MEKK3. Our studies indicated that the SKOV3ip.1 parental line has normal endogenous levels of Tao, Tak1, MLK3 and MEKK3. Future studies, in collaboration with Dr. Rinker-Schaeffer's laboratory will prioritize the testing of this panel of candidate MAP3K's to complete the studies that are now outside the scope of this proposal. Specifically, the effect of depletion of specific candidate MAP3K's will be tested by siRNA depletion, biochemical characterization and testing for reacquisition of metastatic ability *in vivo*.

2. MKK4 metastasis suppression is dependent on it kinase activity. SKOV3ip.1 was transfected with a kinase inactive form of MKK4, the MKK4-KR mutant. This mutant substitutes an arginine for a lysine in the catalytic domain of MKK4 rendering it kinase inactive. A metastasis assay was performed using SKOV3ip.1 with vector control (pLNCX, n=5 clones), HA-MKK4 (n=2 clones) or HA-MKK4-KR (n=10 clones)**.** Cells (1x 10^6) were injected intraperitoneally into SCID mice and the number of metastases at 20 dpi or 30 dpi were compared in pairwise comparisons between SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4 and SKOV3ip.1-HA-MKK4-KR. The results are shown again in **Table 1**. Given the marginal statistical significance (p=0.04), there was some concern that MKK4-KR's metastatic capacity was not entirely different from that of MKK4 and that MKK4 might not be exerting its positive effect on metastasis suppression entirely through its kinase activity. Therefore additional studies were performed to further elucidate whether MKK4's metastasis suppression is dependent on its kinase activity.

Table 1. Pairwise comparison of mean number of metastases *in vivo*: SKOV3ip.1-pLNCX2, SKOV3ip.1-HA-MKK4 and SKOV3ip.1-HA-MKK4-KR.

	Difference	95%CI	p-value
HA-MKK4-HA vs HA-MKK4-KR	-1.28	-2.52, -0.05	0.04
HA-MKK4 vs pLNCX2	-2.13	-3.34,-0.92	0.0004
HA-MKK4-KR vs pLNCX2	-0.85	-1.81, 0.10	0.088

A kinase assay was developed to ensure that the MKK4-KR mutant lacks the catalytic activity necessary to phosphorylate either JNK or p38. **Figure 2** shows that SKOV3ip.1-HA-MKK4 can activate both JNK and p38 but the kinase inactive

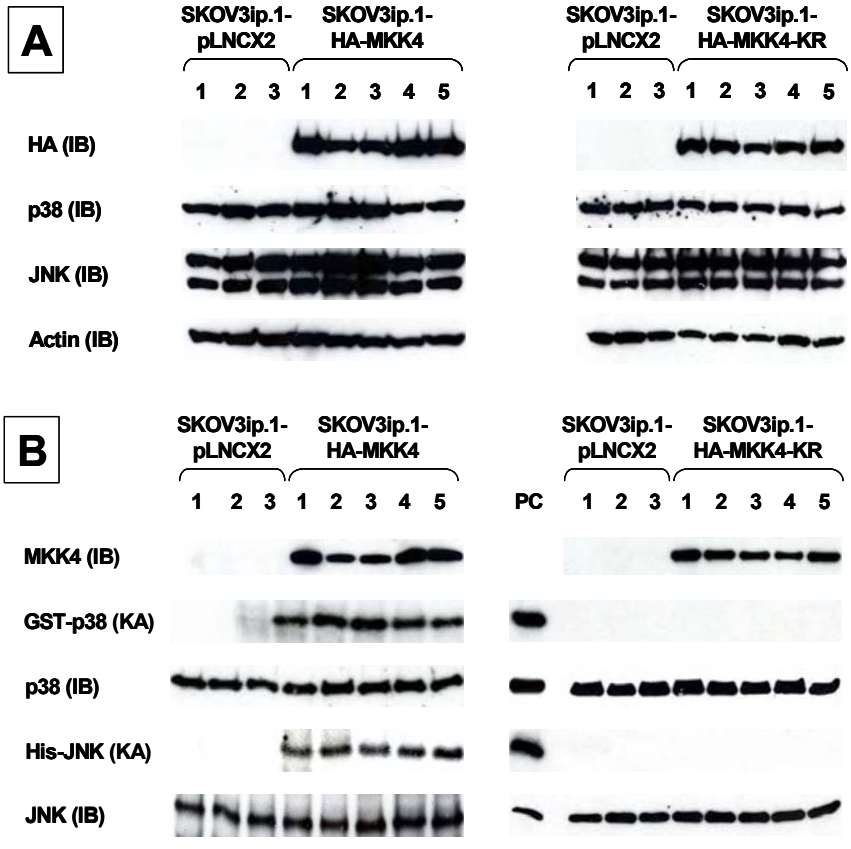
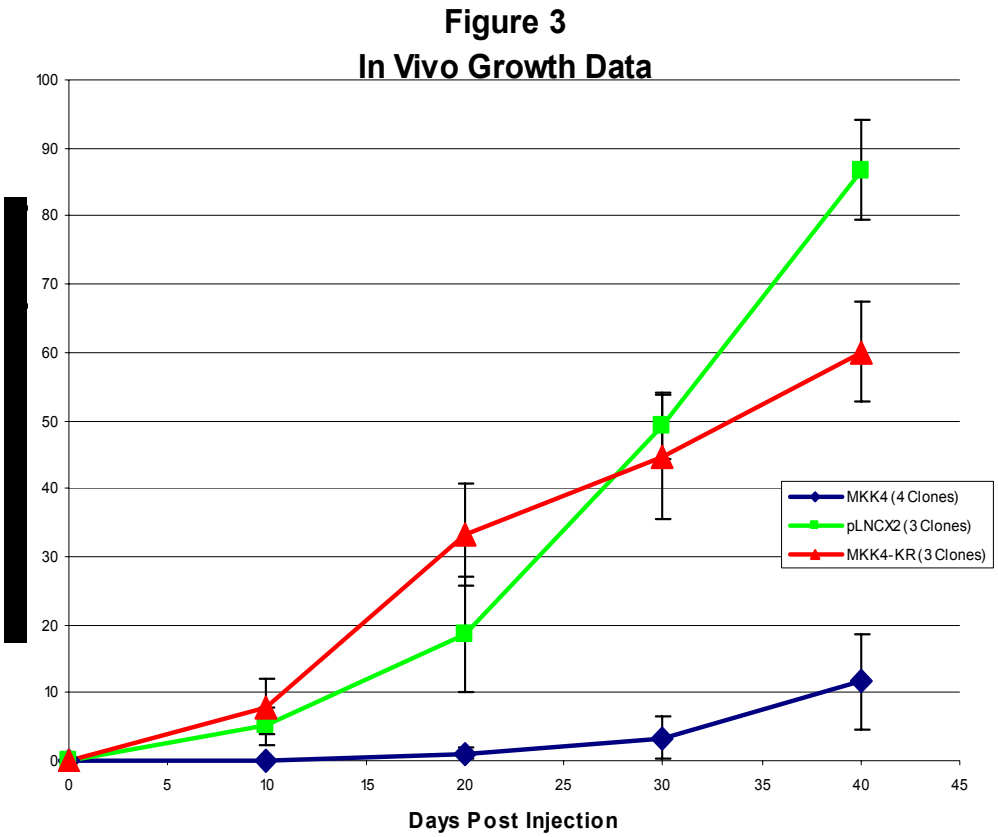


Figure 2: Characterization of SKOV3ip.1-HA-MKK4-KR Clones. (A) Western analysis: lysates of three SKOV3ip.1-pLNCX2 clones and five SKOV3ip.1-HA-MKK4 (left) or SKOV3ip.1-HA-MKK4-KR (right) clones were immunoblotted for the expression of an HA-tagged protein, p38, JNK, and actin. (B) The kinase activity of HA-MKK4 and HA-MKK4-KR in these clones was studied by stimulating the cells with 50 ng/ml anisomycin for 20 minutes and subjecting the lysates to an HA immunoprecipitation. Immunoprecipitates were either immunoblotted for MKK4 or a kinase assay was performed using purified GST-tagged p38 (GST-p38) or purified His-tagged JNK (His-JNK). Immunoblotting for total p38 and JNK served as a loading control.

mutant, SKOV3ip.1-HA-MKK4-KR, does not activate either JNK or p38.



To further clarify the behavior of the kinase inactive MKK4-KR clones as compared to MKK4 *in vivo*, a time course assay was perfomed. Several SKOV3ip.1-HA-MKK4 clones (n= 5 mice per clone, 4 clones) were injected intraperitoneally into mice and compared to 3 SKOV3ip.1-HA-MKK4-KR clones (n=5 mice per clone) and 3 SKOV3ip.1-pLNCX2 clones (n=5 mice per clone). Animals were followed until day 40 to compare biologic behavior of the kinase inactive mutants to the MKK4 clones **(Figure 3).**

Figure 3 (left) shows the similarity in metastatic number at 40 dpi in the SKOV3ip.1-pLNCX2 and SKOV3ip.1-HA-MKK4KR as compared to the small number of metastases in the SKOV3ip.1-MKK4 clones. These data support the concept that MKK4 exerts its effect on metastasis suppression primarily through its kinase activity. These data were published earlier this year in *Cancer Research*.[6]

3. MKK4 mediates metastasis suppression via the JNK and/or p38 pathways. Because MKK4 can activate either the JNK or p38 pathways, it is possible that one or both of these pathways mediated metastasis suppression. We demonstrated in the first year of our award that introduction of MKK6, a potent, unique activator of the MAPK p38 suppressed metastasis by 70%. In the second year of the study, after overcoming problems with stable transfection of SKOV3ip.1 with HA-MKK7, a potent and unique activator of the JNK pathway, we were able to demonstrate that the JNK pathway was not involved in metastasis suppression.

After successful establishment of stable SKOV3ip.1-HA-MKK7 clones, a kinase assay was performed to ensure that endogenous JNK and p38 were present in the SKOV3ip.1-HA-MKK7 clones and JNK was active (**Figure 4A and B**). A standard metastasis assay was performed to compare the mean number of metastases in 5

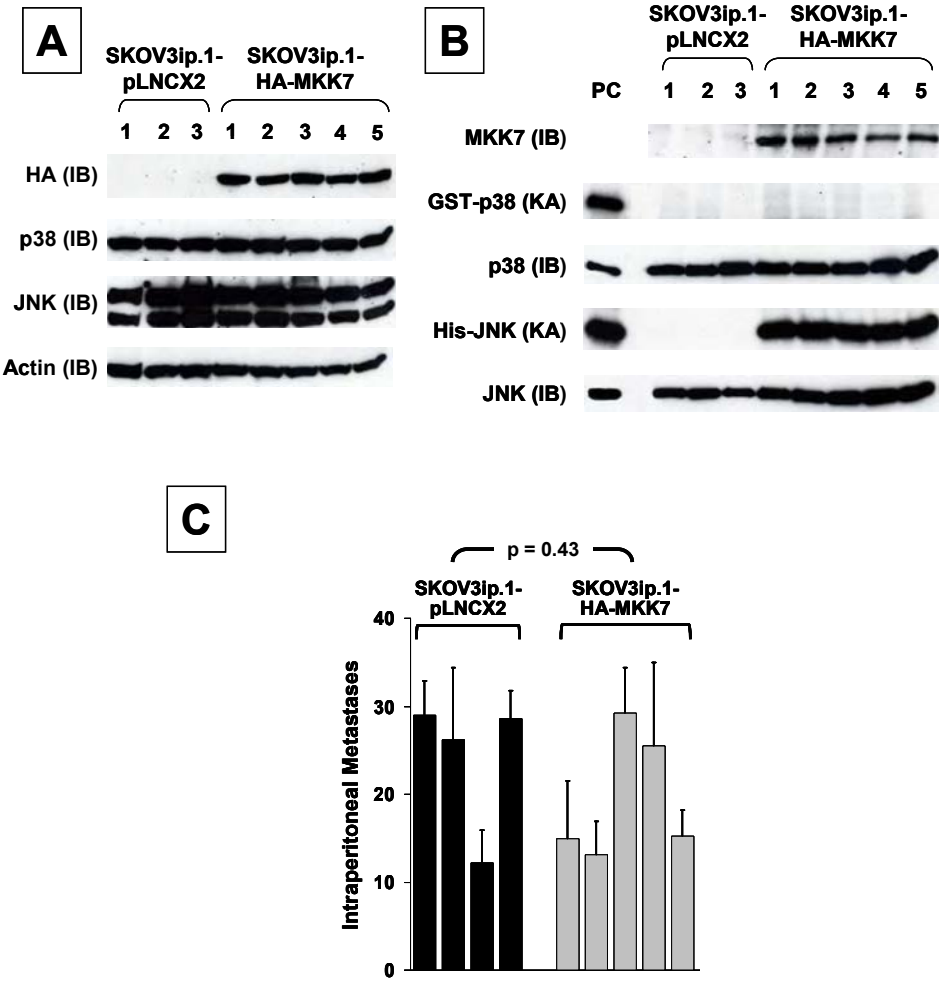


Figure 4. Ectopic Expression of HA-MKK7, a Specific JNK Kinase, Does Not Affect SKOV3ip.1 Metastasis. A) Lysates of three SKOV3ip.1-pLNCX2 clones and five SKOV3ip.1-HA-MKK7 clones were immunoblotted for the expression of an HA-tagged protein, p38, and JNK. Actin was used as a loading control. B) *In vitro* kinase activity of HA-MKK7. Protein lysates from anisomycin-treated clones were subjected to an HA immunoprecipitation. Immunoprecipitates were either immunoblotted for MKK7 or subjected to a kinase assay using purified p38 or JNK. As a positive control (PC), a stimulated SKOV3ip.1-HA-MKK4 clone was used. Immunoblotting for total p38 and JNK served as a loading control. C) Number of metastases formed in mice (*n* = 5 per clone) injected with SKOV3ip.1-pLNCX2 or SKOV3ip.1-HA-MKK7 clones at the experimental endpoint. A pair-wise comparison was determined using a mixed effect model ANOVA: SKOV3ip.1-pLNCX2 vs. SKOV3ip.1-HA-MKK7, p = 0.43.

SKOV3ip.1-HA-MKK7 clones (n=5 mice per clone) to 4 SKOV3ip.1-pLNCX2 clones (n=5 mice per clone) at 20 dpi. Results, shown in **Figure 4C**, demonstrate no significant difference in the mean number of metastases in SKOV3ip.1-HA-MKK7 clones as compared to the vector control clones (19.6 ± 8.8 vs. 24.0 ± 8.6 metastases, respectively, p=0.41). Because MKK7 is a unique and specific activator of JNK, these data suggest that the JNK pathway is not involved in MKK4-mediated metastasis suppression**.** These data have been published along with our data on the importance of MKK4 kinase activity in metastasis suppression.[6]

The second specific aim of our proposal was to analyze the combined effect of chemotherapy and ectopic MKK4 expression on ovarian cancer metastasis. Chemotherapeutic agents typically used in ovarian cancer include paclitaxel and cisplatin. Both these agents activate the JNK pathway. In other cell lines, they have also been shown to activate the p38 pathway. We hypothesized that the combined effect of MKK4 expression and use of chemotherapy *in vivo* would result in prolonged animal survival when compared to either condition alone. We had previously performed a dose finding study to establish the maximally tolerated intraperitoneal dose of cisplatin and paclitaxel chemotherapy. We concluded that the maximally tolerated dose for cisplatin was 4 mg/kg and for paclitaxel, 20 mg/kg q day for 5 days. Our original objective was to determine whether the combined effect of MKK4 expression in conjunction with chemotherapy would prolong animal survival. In designing our experiments, however, we were concerned that treating with chemotherapy at day 20-30 dpi in both groups when there are typically significantly fewer metastases in the MKK4-injected mice (n=3 metastases) as compared to vector control mice (n=23 metastases) would universally eradicate all metastases in the MKK4 mice, thereby not allowing for the ability to see MKK4 effect and interaction with chemotherapy

over time. Therefore, we injected mice with SKOV3ip.1-HA-MKK4 on day 0 or SKOV3ip.1-pLNCX2 on day 30 to allow for equalization of numbers of metastases. On day 54, we injected SKOV3ip.1-HA-MKK4 (n=20 mice) or SKOV3ip.1-pLNCX2 (n=20 mice) with paclitaxel 20 mg/kg q day x 5 or cisplatin 4 mg/kg q week x 2 and sacrificed the mice on day 68 to compare numbers of metastases in the MKK4 groups with or without chemotherapy (**Figure 5**).

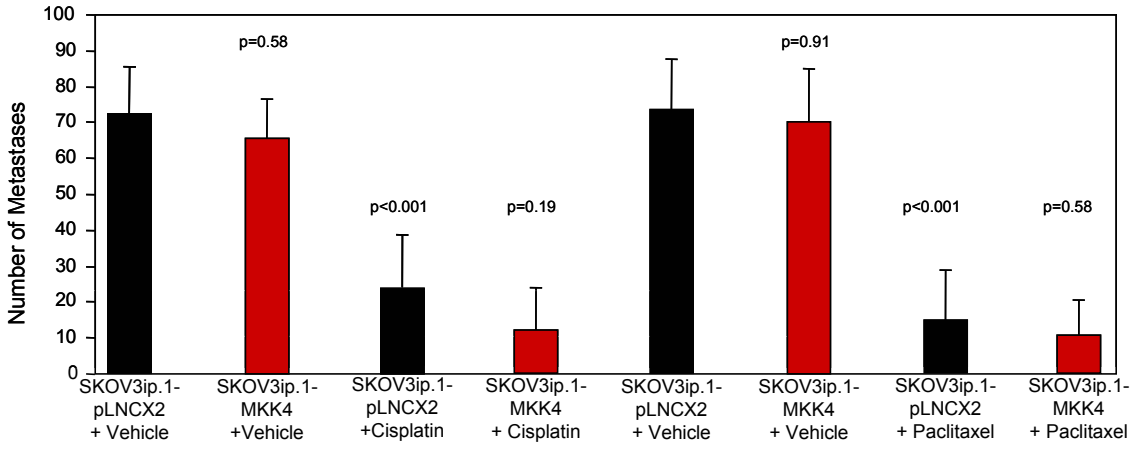


Figure 5. Female nude mice were injected with SKOV3ip.1-HA-MKK4 cells (1 x 10^6) IP on day 0 or with SKOV3ip.1-pLNCX2 (1 x 10^6) IP on day 30. On day 54, animals were treated with cisplatin 4 mg/kg q week x 2 or paclitaxel 20 mg/kg q day x 5. Animals were sacrificed on day 68 and numbers of metastases counted using a square root transformation to stabilize variance. A mixed effects linear model was then employed.

The data in Figure 5 indicate that both cisplatin ($p<0.001$) and paclitaxel ($p<0.001$) significantly reduce the numbers of metastases at day 68. However, there is only a trend toward significance in metastasis suppression in the MKK4 plus cisplatin group ($p=0.19$) over the cisplatin group (SKOV3ip.1-pLNCX2) alone. There is no significant difference in numbers of metastases in the MKK4 plus paclitaxel group as compared to the paclitaxel group alone ($p=0.58$). These data indicate that *when* significant metastases are already established, MKK4 does not augment the effect of chemotherapy on metastatic number. Given our previous data showing that MKK4 exerts its effects early in the process of metastatic colonization, it is possible that MKK4 expression will only augment the effects of chemotherapy during the time when disseminated cells are establishing themselves at secondary sites of disease (i.e. early in the metastatic process).

The second objective within this specific aim was to determine if the combined effect of MKK4 expression and paclitaxel or cisplatin chemotherapy would promote JNK activation over and above either agent alone. We proposed to perform an immunocomplex JNK kinase assay on metastases harvested from animals injected with either SKOV3ip.1-pLNCX2 (vector control) alone, SKOV3ip.1-HA-MKK4 alone, vector control treated with either cisplatin or paclitaxel or SKOV3ip.1-HA-MKK4 treated with cisplatin or paclitaxel. In lieu of performing kinase assays on the metastases, we opted to perform immunoblots on the tissues harvested at day 68 for phospho-JNK and phospho-p38 (**Figure 6**)

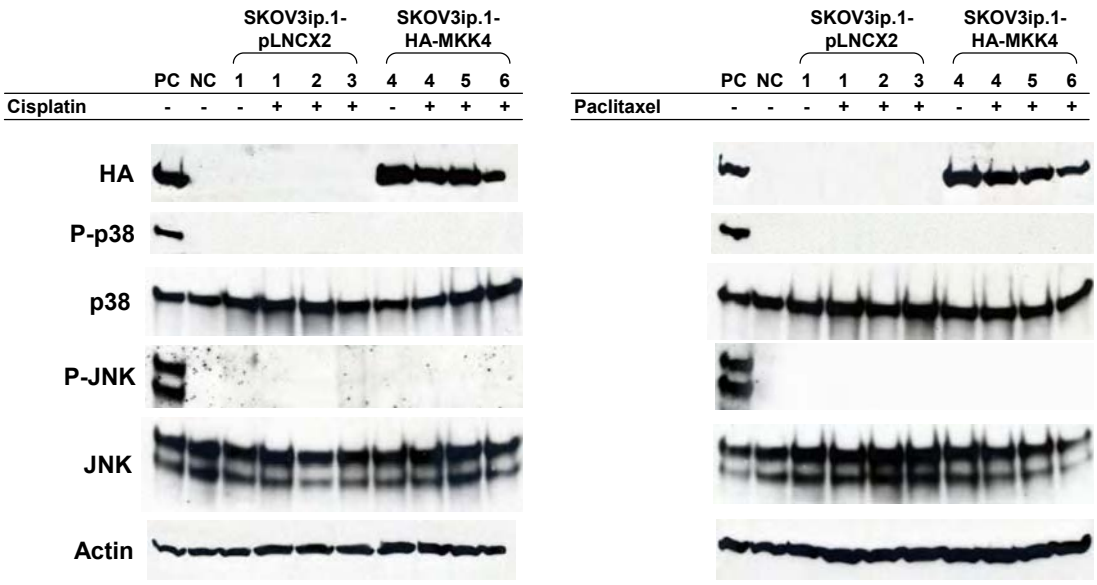


Figure 6. Immunoblot of metastases harvested from female nude nice at day 68 after treatment with (+) or without (-) cisplatin (panel left) and with (+) or without (-) paclitaxel (panel right). PC, positive control, anisomycin treated SKOV3ip.1 cells. NC, negative control, SKOV3ip.1 cells, no anisomycin treatment. Tissue lysates were immunblotted for hemagglutinin (HA) to verify presence of MKK4. Lysates were also immunblotted for phospho-p38 and phospho-JNK. For verification of equal presence of protein, lysates were immunoblotted for total p38, total JNK and actin.

At day 68, there were no differences in phospho-p38 or phospho-JNK levels in the metastases taken from mice injected with SKOV3ip.1-pLNCX2 or SKOV3ip.1-HA-MKK4 treated with either cisplatin or paclitaxel. However, it is of concern to us that there is no detectable phospho-p38 or phospho-JNK in any of the lysates. Therefore, studies are currently underway to confirm, *in vitro*, that there are significant differences in phospho-p38 or phospho-JNK levels after treatment with escalating doses of both cisplatin and paclitaxel.

III. Key Research Accomplishments

- Confirmation that MKK4 suppresses metastasis *in vivo*
- MKK6 suppresses metastasis *in vivo* implicating the p38 pathway in MKK4 mediated metastasis suppression
- MKK7 does not suppress metastasis *in vivo* indicating that MKK4 does not exert its effect on metastasis suppression via the JNK pathway
- MKK4-KR, a kinase inactive form of MKK4 does not suppress metastasis and does not prolong animal survival indicating that MKK4 kinase activity is essential for MKK4-mediated metastasis suppression and biological effect on survival
- MKK4 does not augment the effects on overt metastases over and above the effect of cisplatin or paclitaxel when there are equal numbers of metastases
- At 14 days after treatment with the chemotherapeutic agents cisplatin and paclitaxel, there is no detectable phospho-p38 or phospho-JNK in metastases taken from animals injected with either SKOV3ip.1-HA-MKK4 or SKOV3ip.1-pLNCX2.

IV. Reportable Outcomes

A. Abstracts

1. Jonathan A. Hickson, Yancey Hrobowski, Donald J. Vander Griend, David Benson, Anthony Montag, Theodore Karrison, Dezheng Huo, Joanne Rutgers, Sarah Adams, Carrie Rinker-Schaeffer, S. Diane Yamada. *Mitogen-activated Protein Kinase Kinase 4 (MKK4) Acts as a Metastasis Suppressor Gene in Human Ovarian Carcinoma*, University of Chicago Cancer Biology Retreat, May, 2003.
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B. Manuscripts

1. Hickson JA, Huo D, Vander Griend DJ, Rinker-Schaeffer CW, Tomek R, Karrison T, Yamada SD. The p38 kinases MKK4 and MKK6 suppress metastatic colonization in human ovarian carcinoma. *Cancer Res* 66:2264-2270, 2006.
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V. Conclusions

Over the course of this award, we have been able to verify that MKK4 mediates metastasis suppression *in vivo*. MKK4 effect on metastasis appears to be at the point of metastatic colonization, as metastases expressing MKK4 eventually grow and the animals succumb to metastases. We have been able to dissect out that the p38 pathway and not the JNK pathway is responsible for metastasis suppression *in vivo*. In addition, we have shown that MKK4's kinase activity is essential for this effect. These data identify the p38 signaling pathway as a worthwhile target for upregulation in the management of ovarian cancer. From a therapeutic standpoint, the point at which p38 activity may be most important may be during the steps of metastatic colonization. From a clinical standpoint, it may be meaningful to target the p38 pathway after surgical cytoreduction or after treatment with frontline chemotherapy in the form of consolidation therapy. From our studies, we have not been able to show that MKK4 augments the effects of either cisplatin or paclitaxel on ovarian cancer metastases but from our studies, we conclude that MKK4 may need to be studied for its synergistic effects with chemotherapy at an earlier point in time when the metastatic tumor burden is lower.

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